To Elucidate the Role of Nedd4/Caspase-4/5-GSDM-D Signalling Pathway in Pyroptosis during Atherosclerosis

PhD Synopsis

Scholar	**Salman Shamas**
Enrolment Number	21163CUKmr002
Date of Registration	02 March 2022
Supervisor	**Dr. Abid Hamid Dar** Associate Professor and Head Department of Biotechnology Central University of Kashmir
Co-supervisors	**Dr. Owais Mohmad Bhat** Scientist-D Department of Biotechnology Central University of Kashmir
	Prof. M. Afzal Zargar Professor Department of Biotechnology Central University of Kashmir
	Dr. Nissar Ahmad Wani Scientist-D Department of Biotechnology Central University of Kashmir
Department	**Biotechnology**
School	**Life Sciences**

Central University of Kashmir

Ganderbal, J&K

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1. Background

Atherosclerosis is a chronic inflammatory disease of the inner wall of large and medium-sized arteries. It is initiated by lipid retention, oxidation, and modification, which provoke chronic inflammation and accumulation of fatty and/or fibrous materials in vascular intima, ultimately causing thrombosis or stenosis. Recent understanding of pathogenesis of atherosclerosis explained it as imbalanced lipid metabolism integrated with misconducted immune response leading to chronic inflammatory processes in the vessel wall (1). Recent studies have shown that the pyroptosis that accompanies the inflammatory response may be involved in the atherosclerosis development and progression (2, 3, 4).

Pyroptosis is an inflammatory cell death usually caused by Pathogen-associated molecular patterns (PAMPs) or Damage-associated molecular patterns (DAMPs), associated with activation of inflammasomes and maturation of pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (5). Gasdermin (GSDM) family proteins are the executors of pyroptosis. Caspase mediated generation of N-terminal GSDM-D leads to oligomerization of N-terminal GSDM-D, thereby causing formation of pores across the cell membrane which leads to release of pro-inflammatory cytokines IL-1β ad IL-18 (6). Recent studies have shown that pyroptosis, a type of inflammatory cell death of endothelial cells, macrophages, and smooth muscle cells in the vascular wall can induce plaque instability and accelerate atherosclerosis progression (7, 8, 9).

There are two major pathways for pyroptosis: canonical and noncanonical pyroptosis. Both the pathways use GSDM-D as the downstream effector. Canonical pyroptotic death is mediated by inflammasome assembly, which is accompanied by GSDM-D cleavage, and IL-1β and IL-18 release (10). The assembly of inflammasome initiates due to cytosolic pattern

recognition receptors (PRRs, also known as inflammasome sensors) such as PAMPs and DAMPs (5). Inflammasome is a multiprotein complex of the innate immune response previously described as a regulator of caspase-1 activation and release of the pro-inflammatory cytokines active interleukin IL-1β and IL-18 (11). Stimulation of different cell models with various compounds such as nicotine, oxidized modified low-density lipoprotein (ox-LDL), cholesterol crystals (CC-) and palmitate result in PRRs assemble with pro-caspase-1 and ASC leading to inflammasome formation (12, 13, 14, 15). Nicotine's pro-atherosclerotic properties are essentially brought about by its capacity to increase ROS generation, activate NLRP3 and caspase-1, and finally cause endothelial cells to undergo pyroptosis (12). Ox-LDL activates the NLRP3 inflammasome and increases IL-1 production in human macrophages (13). In mouse immune cells, crystalline cholesterol activates the NLRP3 inflammasome, which leads to the progression of atherosclerosis (14). Early hyperlipidaemia enhances EC activation prior to monocyte recruitment via a caspase-1-Sirt1-Activator protien-1(AP-1) mechanism, which then leads to the initiation of atherosclerosis (15). Active-caspase-1 cleaves GSDM-D into N-terminal GSDM-D (16). GSDM-D, encoded by GSDMD on chromosome 8 (8q24.3), comprises of 31 kDa N-terminus (GSDMD-N) and a 22 kDa C-terminus (GSDMD-C) connected by a peptide linker. Upon activation, the linker is cleaved to separate GSDMD-N from its autoinhibitory domain, GSDMD-C. GSDMD-N forms a transmembrane pore that releases cytokines such as interleukin IL1β and IL-18 and also disturbs the regulation of ions and water, eventually resulting in pyroptosis. In addition, IL-1β and IL-18 are secreted from the pores formed by GSDMD-N (17). Alternatively, pyroptosis can be induced via the non-canonical pathway by the intracellular detection of lipopolysaccharides (LPS), oxidized phospholipids, palmitate which activates caspases -4, 5, and/or 11 (mouse caspase-11) to cleave GSDM-D (5, 17, 18, 19, 20, 21, 22, 23). Cytosolic LPS is detected by the cysteine protease caspase-4 and caspase-5, which oligomerizes and proteolytically activates the cell death-

inducing protein GSDM-D upon direct attachment to LPS (17). A recent study in macrophages found that treatment with *Francisella novicida* caused Caspase-4 –mediated GSDM-D dependent pyroptosis and NLRP3 inflammasome activation. Caspase-4 promotes inflammasome responses to *F. novicida* infection in human macrophages (18). During myocardial reperfusion, caspase-4 mediated cleavage of GSDM-D promoted pyroptosis in the heart and Cardiac microvascular endothelial cells (CMECs) which was suppressed by beclin1 overexpression that promoted autophagic flux (19). A recent study showed that Caspase-4 mediated pulmonary artery endothelial cell pyroptosis contributes to pulmonary arterial hypertension, knockdown of caspase-4 in these cells prevented the onset of TNF-induced pyroptosis, and also inhibited the activation of the pyroptosis effectors GSDM-D and GSDM-E (20). Another study demonstrated that overexpression of the caspase-5 gene activated pyroptosis and also reduced cell viability to alleviate the functions of agomiR-527 in LPS-treated human gingival fibroblasts (21). In tubular epithelial cells, caspase-4/5-mediated pyroptosis plays a critical role in the toxic contrast media's host-defence and immune surveillance activities. Caspase-4/5 expression is essential for the activation of epithelial pyroptosis in human and tubular epithelial cells, which contributes to the development of Contrast-induced acute kidney damage (23). Caspase-4/5/11-mediated pyroptosis releases IL-1α and high mobility group box 1 (HMGB1) in hepatocytes. These cytokines along with other alarmins are secreted through these cell membrane pores or after membrane lysis, which may cause the aggregation of immune cells and chronic inflammation (22). Thus caspases such as caspase-1, 4 and 5 in Homo sapiens, and caspase-11 in murine plays an important role in pyroptotic pathway mediated via GSDM-D. All these studies provided that caspase-4/5 and GSDM-D play an important role in pyroptotic pathway under *in vitro* and *in vivo* conditions. However, this project is focussed on endothelial cells and will try to explore whether caspase-

4/5-GSDM-D contributes to pyroptosis during the development and progression of atherosclerosis.

Ubiquitination is an important post-translational protein modification and plays a crucial regulatory role in regulating inflammatory cells (24). Protein ubiquitination process is sequentially mediated by three enzymes: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), which controls substrate specificity. E3 enzymes are divided into three groups: homologous to E6-AP COOH terminus (HECT), really interesting new gene (RING), and U-box E3s. In the case of HECT E3s, ubiquitin is first transferred to the active cysteine of E3, and the E3 subsequently adds it to the substrate. The other E3s directly mediate transfer of ubiquitin from E2 to the substrate (25). One such HECT E3s known as Neural precursor cell-expressed developmentally down regulated gene 4 (Nedd4) is characterized by an N-terminal C2 domain, 3–4 tryptophan repeats and a C-terminal HECT domain (26). It is widely expressed in mammalian tissues and plays distinct roles in different cellular functions. Nedd4 can target different substrates such as phospho-AKT, insulin like growth factor 1 receptor, and phosphatase and tensin homolog (27). Furthermore, Nedd4 has been found to have important regulatory roles in adaptive immunity mediating the ubiquitination and subsequent degradation of Cbl-b to promote the conversion of naive T cells into activated T cells (28). Ubiquitination of TRAF3 by Nedd4 promotes CD40-mediated AKT activation in B cells (29). It has also been found that Nedd4 inhibits AP-1 activation and TNF-α production via targeting p38α for polyubiquitination in macrophages (30). A recent study has shown Nedd4 also controls non-canonical inflammasome activation through the polyubiquitination and subsequent degradation of caspase-11/4 in RAW264.7 macrophages (31). Nedd4 directly interacts and mediates K48-linked polyubiquitination of caspase-11/4 at multiple K residues and subsequent degradation through the 26S proteasome in immortalized

bone marrow-derived macrophages. Furthermore, this study also showed that Nedd4 protein levels were decreased by over expression of caspase-11 or caspase-4 (30).

Endothelial dysfunction contributes mainly in CVDs, particularly in atherosclerosis. Blood vessels are lined with a single layer of endothelial cells (ECs), which create a dynamic barrier between the blood and underlying tissue. The structural and functional integrity of ECs is essential for the physiological function of blood vessels, and their altered function plays a pivotal role in the pathogenesis of human diseases in particular CVDs such as atherosclerosis. Cell adhesion molecules (CAMs) are the key mediators of endothelial barrier function (32). CAMs mediate cell-cell, cell-matrix adhesion, and transmit signals across the plasma membrane (33). Cadherins, integrins, selectins and immunoglobulin- (Ig) like cell adhesion proteins (Ig-CAMs) are major CAMs present in the human genome. Ig-CAMs are cell surface glycoproteins with one or more Ig domains in the extracellular domain, a single transmembrane domain and a C-terminal intracellular domain. The Ig-containing extracellular domain of Ig-CAMs commonly mediates homophilic interaction by binding to the same structure on an opposing cell surface, or other cell surface receptors via heterophilic dimerization. The C-terminal intracellular domains, interact with signalling proteins that regulate cell morphology and other cellular functions (34). Ig containing and proline-rich receptor-1 (IGPR-1) is a newly identified Ig-CAM expressed by epithelial and endothelial cells. IGPR-1 is a transmembrane glycoprotein that consists of an extracellular domain with a single Ig domain, a single transmembrane domain, and a highly conserved intracellular domain (35). The C-terminal intracellular domain is highly enriched in proline followed by serine residues that have the potential to undergo phosphorylation. The intracellular domain of IGPR-1 interacts with multiple signalling proteins containing a Src homology 3 (SH3) domain, includingSPIN90/WISH to mediate the biological function of IGPR-1 (36). IGPR-1 expression is highly conserved in higher mammals, but no obvious homolog of IGPR-1 is found in mouse

or rat. IGPR-1 is expressed in the endothelial cells of vein and arteries and regulates angiogenesis, endothelial barrier function and response to shear stress (35). IGPR-1 was recently identified as a novel CAM expressed in ECs which contributes to EC barrier function, however its role in the development and progression of atherosclerosis has not yet been elucidated. Recent studies demonstrated that NEDD4 plays pivotal roles in regulating IGPR-1 ubiquitination, downregulation, and stability. NEDD4 promotes both K48- and K68-dependent polyubiquitination of IGPR-1 stimulating endocytosis via lysosomal degradation of IGPR-1 in HEK-293 cells and in multiple endogenously IGPR-1 expressing human skin melanoma cell lines (37). Since, IGPR1 belongs to cell adhesion molecule family that plays a crucial role in endothelial dysfunction and contributes to atherosclerosis. Thus, it is worth to explore role of IGPR1 and its regulation by Nedd4 in endothelial cells and their dysfunction. Therefore, the present study hypothesised that NEDD4/Caspase-4/5-GSDM-D pathway regulates pyroptosis in endothelial cells during atherosclerosis.

2. Review of Literature

In recent decades, atherosclerosis is considered as one of the main vascular disease associated with CVDs which is developed by the accumulation of fatty and/or fibrous materials in vascular intima. Atherosclerosis is a chronic inflammatory condition of the arteries that accounts for approximately 50% of all fatalities in Westernized societies. Endothelial dysfunction is the basic underlying cause of vascular diseases which initiates atherosclerosis and followed by a cascade of events (accumulation of lipids, fibrous elements, and calcification), triggers the vessel narrowing and activation of inflammatory pathways. It is a major reason for the development of vascular diseases and a leading contributor to death around the world, about 7.2 million deaths each year, and the prevalence is expected to increase about 18% by 2030 (38). Atherosclerotic cardiovascular disease primarily affects the heart and brain, resulting in ischemic heart disease (IHD) and ischemic stroke. IHD and stroke are the world's

first and fifth leading causes of mortality, respectively. According to the 2015 Global Burden of Diseases, Injuries, and Risk Factors Study, cardiovascular disease (CVD) affected an estimated 422.7 million people and caused an estimated 17.9 million deaths globally in 2015, accounting for 31% of all global fatalities. It has been reported that plaque rupture causes 75% of acute myocardial infarctions, with males over 45 years having the highest incidence, while women have a higher prevalence after the age of 50.

India has one of the world's highest burdens of CVDs. The yearly number of CVD fatalities in India is expected to have rose from 2.26 million in 1990 to 4.77 million in 2020. In 2016, India recorded 63% of total fatalities owing to non-communicable diseases, with CVDs accounting for 27%. CVDs are also responsible for 45% of mortality in the 40-69 age range in India. Nearly a quarter (24.8%) of all fatalities in India, according to age-standardized estimates from the Global Burden of Disease study (2010), are related to CVD. India has a higher age-standardized CVD mortality rate than the rest of the world, with 272 per 100,000 people, compared to 235 per 100,000 people globally. In 2016, the estimated prevalence of CVDs in India was estimated to be 54.5 million. According to a few population-based surveys, the prevalence of CVD was estimated to be 3-4% in rural India in 2003, and 8-10% in urban areas. To create and implement an effective strategy for the prevention and treatment of CVD in people, a more thorough understanding of a wide variety of CVD risk factors and the mechanisms related to them is required.

Recent Studies have shown that pyroptosis of endothelial cells, macrophages, and smooth muscle cells in vascular wall can induce plaque instability and accelerate atherosclerosis progression (12, 13, 14). Pyroptosis is a newly discovered pattern of programmed cellular necrosis, characterized by cell swelling, plasma membrane rupture, and massive production and release of pro-inflammatory factors, triggering a cascade of amplified inflammatory responses, often induced by endogenous injury or bacterial and viral infections

(39). Various stimuli such as nicotine, hyperlipidaemia, oxidized modified low-density lipoprotein (ox-LDL), cholesterol crystals (CC) and palmitate promote the development of atherosclerosis and can induce the pyroptosis-associated inflammasome and caspases pathway activation through different pathways. The molecular mechanisms of pyroptosis mainly include caspase-1-dependent canonical pathway and caspase-4/5/11-dependent non-canonical pathway.

In the caspase-1 dependent pyroptosis pathway, cells can activate inflammatory vesicles to trigger pyroptosis in response to multiple factors activating the respective inflammasomes (including NLRP3, AIM2 or Pyrin) through the action of PAMPs and DAMPs (40). These inflammasomes are innate immune system receptors and sensors that regulate the activation of caspase-1 and induce inflammation in response to infectious microbes and molecules derived from host cell (11). Inflammasomes such as NLRP3 recruits ASC and pro-caspase-1 through various pathways, causing activation of caspase-1 which in turn activates GSDM-D and regulate the maturation and secretion of pro-inflammatory cytokines such as IL-1β and IL-18 (41).

GSDM-D is a protein which is composed of a 31 kDa N-terminus (GSDMD-N) and a 22 kDa C-terminus (GSDMD-C) connected by a peptide linker. Upon activation, the linker is cleaved to separate GsdmD-N from its auto inhibitory domain, GSDM-C. GSDM-N then triggers the rupture of cell membranes and promotes the release of inflammatory factors, cell swelling and pyroptosis (42).

Unlike canonical signalling pathway, caspase-4/5/11-mediated pyroptosis releases IL-1α and high mobility group box 1 (HMGB1). These cytokines along with other alarmins are secreted through cell membrane pores or after membrane lysis, which may cause the aggregation of immune cells (43). In contrast to caspase-1, which cleaves pro-IL-1β/IL-18 and GSDM-D, activated caspases- 4/5/11 has been considered to recognize GSDM-D and cleave

at D275 of human GSDM-D or D276 of murine GSDM-D for generating pyroptosis pores (44). Caspase-4/5/11 regulate the secretion of IL-1β by activating the NLRP3/ASC/caspase-1 pathway instead of maturing pro-IL-1β directly (45). Non canonical pathways are mostly activated by lipopolysaccharides but recent studies have shown that they are also activated by DAMPs such as oxidized phospholipids (oxPAPC), heme and palmitate (46, 47, 48).

Caspases-4 and -5 are the human orthologues of murine caspase-11, sharing with it 68% and 47% amino acid sequence identity respectively (49). A recent study has shown that Caspase-11-GSDM-D mediated pyroptosis and the subsequent proinflammatory response in macrophages participate in the pathogenesis of atherosclerosis (50). It has also been seen that activation of Caspase-11/4/5 triggers not only Caspase-1 independent pyroptosis but also Caspase-1-dependent release of the pro-inflammatory cytokines IL-1β and IL-18 (51). A recent study has showed that the increased expression of caspase-4/5 in human ECs in sepsis is required for activation of endothelial pyroptosis, thereby contribute to the development of fulminant pulmonary edema and acute lung injury (52). Another study showed that human monocytes from obese patients have elevated caspase-4/5 activation and fatty acids that trigger inflammatory response and consequent release of IL-1β/IL-18 (47).

Endothelial cells contain the inner surface of blood vessels, maintain vascular and tissue homeostasis, and regulate many important physiological processes, including angiogenesis, immune responses, and metabolic dynamics including fluid exchange in all organs. Highly specific molecular interactions, often via cell surface receptors known as cell adhesion molecules (CAMs), e.g. integrins and cadherins, are involved in the conversion of mechanical forces into biochemical signals to control a variety of biological processes (33). Immunoglobulin and proline-rich receptor-1 (IGPR-1, also known as TMIGD2) is a newly identified CAM that plays an important role in endothelial cell adhesion (36). Unlike the classical cadherins and tight junction proteins such as JAMs, claudins family proteins, and

occludin, IGPR-1 expression is restricted to higher mammalians as it is not present in rodents such as mouse or rat (32). IGPR-1 is composed of three major domains: extracellular, transmembrane, and intracellular. The extracellular domain of IGPR-1 contains a single Ig domain followed by a single transmembrane domain and a proline rich intracellular domain. The Ig-containing extracellular domain is required for IGPR-1 to mediate endothelial cell–cell interaction and barrier function. IGPR-1 is localized to adherens junctions and, is activated through trans-homophilic dimerization. A recent liquid chromatography–tandem mass spectrometry analysis of IGPR-1 identified seven phosphorylated serine residues on the cytoplasmic domain of IGPR-1, including Ser186, Ser220, Ser238, Ser243, Ser249, Ser262, and Ser266, five of which are located in the proline-rich region. Although the functional importance of these phosphorylation sites remains to be determined, phosphorylation of Ser220 is regulated by homophilic trans-dimerization of IGPR-1 and is required for endothelial barrier function and angiogenesis (35).

Neural precursor cell-expressed developmentally down regulated protein 4 (Nedd4) is an E3 ubiquitin ligase which regulates target protein stability and cellular localization via proteosomal degradation. The Nedd4 protein has a modular structure that is shared among the Nedd4 family, consisting of an amino-terminal C2 calcium- dependent phospholipid binding domain, 3-4 ww protein-protein interaction domains ad a carboxyl-terminal catalytic HECT ubiquitin ligase domain. The WW domain interacts with proline rich PPxY motifs in target proteins to mediate interactions with substrates ad adaptors. The human Nedd4 gene is located on chromosome 15q21.3, and the protein is 120 kDa (53). In the recent years the association between Nedd4 and CVDs has been focused by many investigators. Accumulating evidence show that Nedd4 is involved in regulating the epithelial sodium channel ENaC activity (54). Recent studies have shown that Nedd4 is involved in various pathophysiological processes ad exert distinct influences on various CVDs including hypertension, dilated cardiomyopathy,

diabetic cardiomyopathy, myocardial infarction, heart failure, etc. (55, 56, 57, 58). Another study showed that Nedd4 is involved in the degradation of non-canonical caspases such as 11/4/5 (30). It has also been shown that Nedd4 is involved in the degradation of IGPR-1 (37). Therefore in the present study we will investigate the role of Nedd4/IGPR-1/GSDM-D mediated signalling in pyroptosis during atherosclerosis.

3. Identification of Research Gaps

Atherosclerosis is the leading cause of mortality in the developed world, while risk factor modifications has improved results over the last decade, there is still no permanent solution. Recent research suggests that the initiation and progression of atherosclerosis is mostly influenced by cell death and inflammation. Pyroptosis is a newly discovered programmed cell death linked with an inflammatory response that plays an important role in the formation and progression of atherosclerosis by activating several signalling pathways. The death of vascular cells such as ECs, macrophages, and smooth muscle cells (SMCs) is prevalent throughout the development of atherosclerosis, and pyroptosis is believed to widely occur in all these cells. However, the role of Caspases-4 and 5 in the pyroptosis during the development of atherosclerosis has not been elucidated yet. Although, caspase-4/5 have shown important role in diseases like sepsis, cancer, fulminant pulmonary edema, acute lung injury, chronic kidney disease (CKD) and obesity but its role in cardiovascular diseases such as atherosclerosis is yet to be elucidated. Also, the defined mechanism for the activation of inflammasomes by non-canonical caspases has not been discovered yet. In addition, Nedd4 is involved in many vascular diseases such as vascular calcification, hypertension, pulmonary hypertension and myxomatous mitral disease. It has also been shown that Nedd4 is involved in the degradation of non-canonical caspases such as 11/4/5, however its role in regulating pyroptosis during atherosclerosis has not been elucidated yet. Therefore, the present study will investigate the role of Nedd4/Caspase-4/5-GSDM-D signalling pathway in pyroptosis during atherosclerosis.

4. Aim

The aim of the present study is to investigate whether the Nedd4/ Caspase- 4/5-GSDM-D mediated signalling pathway regulates pyroptosis in endothelial cells during atherosclerosis.

5. Objectives

Specifically, the proposal will focus on the following objectives:

1. To check expression of IGPR-1 and Nedd4 in palmitate stimulated endothelial cells in pyroptosis during atherosclerosis.
2. To study the role of caspase-4/5 mediated pyroptosis in palmitate stimulated endothelial cells in atherosclerosis.
3. To determine the role of Non-canonical GSDM-D-NLRP3 pathway in endothelial cells during pyroptosis in atherosclerosis.
4. To elucidate the role of Nedd4/IGPR-1 signalling in endothelial cells during pyroptosis in atherosclerosis.

6. Methodology

Aim 1: To check expression of IGPR-1 and Nedd4 in palmitate stimulated endothelial cells in pyroptosis during atherosclerosis.

Approach

a. **Cell Culture and lipid deposition:**

Human umbilical vein endothelial cells (HUVECs) will be cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin in humidified 100% air at 37 °C with 5% CO_2. Confluent cell cultures of HUVECs will be treated with ox-LDL (100 µg/ml) (59) and palmitate (50 µg/ml)(60) for 24 h respectively. At the end of the experiment, the cells will be stained

with 0.5% Oil Red O solution for 1h, and then differentiated in a 60% propylene glycol solution for 5 min to confirm the lipid deposition (61).

b. Pyroptosis cell death assay:

Pyroptotic cell death will be evaluated with lactate dehydrogenase (LDH) release. For this, cell culture supernatants will be collected, and LDH activity will be detected using the LDH assay kit. Briefly, 25 µl cell supernatant and 25 µl substrate will be mixed together and incubated at 37°C for 15 min. Then, 25 µl of 2, 4-dinitrophenylhydrazine will be added into the samples and incubated at 37°C for 15 min. Finally, 250 µl of 0.4 mol/L NaOH solution will be added and incubated with the mixture at room temperature for 5 min. Absorbance will be measured at 450 nm on a spectrophotometric microplate reader (44).

c. Scanning electron microscopy:

The morphology of the pyroptotic cells will be analysed at high resolution by scanning electron microscopy. Cells will be fixed with 2.5% glutaraldehyde overnight, and then rinsed with PBS three times. Samples will be dehydrated through a graded series of ethanol (30%, 50%, 70%, 95% and 100%) and then dried by tertiary butanol method. Dried specimens will then be sputter coated with gold-palladium and imaged with scanning electron microscope (62).

d. Caspase-4/5 colorimetric assay:

Caspase-4/5 colorimetric assay kit will be purchased from R&D Systems, Inc. This assay will be carried to check the enzymatic activities of caspase- 4/5. Cells will be suspended in 50 µl of chilled cell lysis buffer and incubated on ice for 30 min. Cell extracts will be centrifuged at 10,000 × g for 10 min at 4°C. Cytosolic proteins will be assayed, and 100-200 µg protein will be diluted with cell lysis buffer. And then 50 µl 2 × Reaction Buffer (containing 10 mM DTT) will be added to each sample. Ac-LEVD-pNA (4 mM) will be added and incubated at 37°C for 4 hr. The plates will be read at

405 nm. The caspase-4/5 activity will be indirectly measured by monitoring cleavage of Ac-LEVD-pNA substrates (63).

e. Quantitative RT-PCR analysis:

RT-PCR will be performed to determine the mRNA expression of Nedd4 and IGPR-1, using gene specific primers. The primer sequences that will be used are as follows:

Nedd4: Forward 5′-TTGCACTTTGCAGCCAGAAG -3′,

Reverse 5′-CTGTCTGGGAGTCAGCTTCA -3′

IGPR-1: Forward 5′-GACCCGAATTCAGAAACAC -3′,

Reverse 5′-GCCCTTCTCAAAACGTA -3′

Briefly, total mRNA will be extracted using TRIzol reagent and then reverse transcribed into cDNA. PCR amplification will be performed with SYBR Green PCR Master Mix. GAPDH will be used as an internal control. Data will be presented as fold increase of target genes normalised to the housekeeping gene, GAPDH (64).

f. Western Blotting analysis:

Total cell lysates will be prepared and micro-centrifuged. Proteins in the supernatant will be extracted, fractionated using 12% SDS-PAGE, and transferred onto PVDF membranes (Millipore). Membranes will be blotted with primary antibodies against NEDD4 and IGPR-1 at 4°C over-night followed by incubation with fluorescence-conjugated anti-rabbit IgG secondary antibodies for 1 h at room temperature. The chemiluminescence technique using LI-COR Odyssey Fc will then be used for identification of bands, and the band intensity of target proteins. GAPDH will be used as a loading control (65).

Aim 2: To study the role of caspase-4/5 mediated pyroptosis in palmitate stimulated endothelial cells in atherosclerosis.

Approach

a. Pharmacological inhibition and silencing of caspase-4/5:

To determine the role of caspase-4/5 in atherosclerosis, firstly, HUVECs will be treated with the Caspase-4/5 pharmacological inhibitor (Z-LEVD-fluoromethylketone). Also, caspase 4/5 will be inhibited at genetic level using siRNA. The siRNA will be used to knockdown the expression of Caspase-4/5 in these endothelial cells.

The following validated siRNAs will be used:

Caspase-4 S1, 5′-GUGUAGAUGUAGAAGAGAATT-3′ and caspase-4 S2, 5′-CCUAGAGGAAGAUGCUGUUTT-3′

Caspase-5 S1, 5′- AUAGAACGAGCAACCUUGACAA -3′ and caspase-5 S2, 5′-CUACACUGUGGUUGACGAAAA -3′

Cells will be nucleofected with 300 nM siRNA according to the manufacturer's instruction. Transfected HUVECs will be stimulated with ox-LDL (100 μg/ml) (59) and palmitate (50 μg/ml) (60) for 24 h respectively followed by transcriptional and translational expression of caspase-4/5 and GSDM-D.

b. Quantitative RT-PCR:

RT-PCR will be performed to determine the mRNA expression of caspase- 4/5, and GSDM-D using gene specific primers.

CASP4: Forward 5′-AAGAGAAGCAACGTATGGCAGGAC-3′,

Reverse 5′-GGACAAAGCTTGAGGGCATCTGTA-3′

CASP5: Forward 5-GGTGAAAAACATGGGGAACTC-3,

Reverse 5′-TGAAGAACAGAAAGCAATGAAGT-3′

GSDM-D: Forward 5′- GTGTGTCAACCTGTCTATCAAGG-3′

Reverse 5′-CATGGCATCGTAGAAGTGGAAG-3′

GAPDH: Forward 5′-GTCTCCTCTGACTTCAACAGCG -3′

Reverse 5′-ACCACCCTGTTGCTGTAGCCAA-3′

Briefly, total mRNA will be extracted using TRIzol reagent and then reverse transcribed into cDNA using cDNA synthesis kit. PCR amplification will be performed with SYBR Green PCR Master Mix. GAPDH will be used as an internal control. Transcript levels relative to GAPDH will be calculated using the ΔC_t method (64).

c. Western Blotting analysis:

Total cell lysates will be prepared and micro-centrifuged. Proteins in the supernatant will be extracted, fractionated using 12% SDS-PAGE, and transferred onto PVDF membranes (Millipore). Membranes will be blotted with primary antibodies against caspase-4/5 and GSDM-D at 4°C over-night followed by incubation with fluorescence-conjugated anti-rabbit IgG secondary antibodies for 1 h at room temperature. The chemiluminescence technique using LI-COR Odyssey Fc will then be used for identification of bands, and the band intensity of target proteins. GAPDH will be used as a loading control (65).

d. ELISA for measurement of pro-inflammatory cytokine IL-1β and IL-18:

During pyroptosis, the cells secrete pro-inflammatory cytokine IL-1β and IL-18 via GSDM-D. Hence, in this experiment we will measure concentration of the pro-inflammatory cytokine IL-1β and IL-18 in the supernatant by ELISA according to the manufacturer's instructions. The optical density (OD value) of each well will be detected by microplate reader at 450 nm wavelength.

Aim 3: To determine the role of Non-canonical GSDM-D-NLRP3 pathway in endothelial cells during pyroptosis in atherosclerosis.

Approach

a. Cell Culture and lipid deposition:

Human umbilical vein endothelial cells (HUVECs) will be cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin in humidified 100% air at 37 °C with 5% CO2. Confluent cell cultures of HUVECs will be treated with ox-LDL (100 µg/ml) (59), palmitate (50 µg/ml) (62) and GSDM-D inhibitor, Disulfiram (5 µmol/L) for 24 h (63). The cells will be stained with 0.5% oil Red O solution for 1 h, and then differentiated in a 60% propylene glycol solution for 5 min to confirm the lipid deposition.

b. Quantitative Real-Time PCR analysis:

RT-PCR will be performed to determine the mRNA expression of GSDM-D, NLRP3, ASC and Caspase-1 using gene specific primers. The primer sequences for each gene will be as follows:

GSDM-D: Forward 5′- GTGTGTCAACCTGTCTATCAAGG-3′

Reverse 5′-CATGGCATCGTAGAAGTGGAAG-3′

NLRP3: Forward 5′-ATTACCCGCCCGAGAAAGG -3′,

Reverse 5′-TCGCAGCAAAGATCCACACAG -3′

ASC: Forward 5′- CCCATAGACCTCACTGATAAAC-3′

Reverse 5′-AGAGCATCCAGCAAACCA -3′

Caspase-1: Forward 5′- AGGAGGGAATATGTGGG-3′

Reverse 5′-AACCTTGGGCTTGTCTT -3′

Briefly, total mRNA will be extracted using TRIzol reagent and then reverse transcribed into cDNA. PCR amplification will be performed with SYBR Green PCR Master Mix. GAPDH will be used as an internal control. Data will be presented as fold increase of target genes normalised to the housekeeping gene, GAPDH (64).

c. Western Blotting analysis:

Total cell lysates will be prepared and micro-centrifuged. Proteins in the supernatant will be extracted, fractionated using 12% SDS-PAGE, and transferred onto PVDF membranes (Millipore). Membranes will be blotted with primary antibodies against GSDM-D, NLRP3, ASC and Caspase-1 at 4°C over-night followed by incubation with secondary antibodies for 1 h at room temperature. The chemiluminescence technique using LI-COR Odyssey Fc will then be used for identification of bands, and the band intensity of target proteins. GAPDH will be used as a loading control (65).

d. Inflammasome formation:

Confocal Microscopy:

To check inflammasome formation, HUVECs will be grown on the cover slides. The slides will be fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min. After this step, cells will be rinsed with PBS and incubated with primary anti-NLRP3 and anti-ASC or anti-caspase-1 primary antibodies overnight at 4° C. Next day, cells will be washed with PBS three times and then incubated with Alexa-488- or Alexa-555-labeled secondary antibodies for 1 h at room temperature. Finally, the slides will be mounted by DAPI-mounting solution and then analysed by confocal laser scanning microscope. The photos will be taken, and then colocalization of NLRP3 with ASC or caspase-1 will be analysed by the Image-Pro plus 6.0 software. The summarized data of colocalization efficiency will be expressed as Pearson correlation coefficient (PCC) (66).

Inflammasome activation:

To check inflammasome activation, we will perform caspase-1 colorimetric assay in cell lysate to analyse the caspase-1 activity. Also, we will perform ELISA to measure IL-1β and IL-18 levels in cell supernatant.

Caspase-1 colorimetric assay:

Caspase-1 colorimetric assay kit will be purchased from R&D Systems, Inc. briefly, cells will be suspended in 50 µl of chilled cell lysis buffer and incubated on ice for 30 min. Cell lysate suspension will be centrifuged at 10,000 × g for 10 min at 4°C. Cytosolic proteins will be quantified and 100–200 µg protein will be diluted in cell lysis buffer. And then 50 µl 2 × reaction buffer (containing 10 mM DTT) will be added to each sample. YVAD-pNA substrates (4 mM) will be added and incubated at 37°C for 4 hr. The plates will be read at 405 nm. The caspase-1 activity will be indirectly measured by monitoring cleavage of YVAD-pNA substrates (67).

e. **ELISA for measurement of pro-inflammatory cytokine IL-1β and IL-18**:

The secreted levels of IL-1β and IL-18 will be measured in the HUVECs supernatants using Human IL-1β and IL-18 ELISA kit respectively according to the manufacturer's instructions.

AIM 4: To elucidate the role of Nedd4/IGPR-1 signalling in endothelial cells during pyroptosis in atherosclerosis.

Approach

a. **Cell culture and treatment:**

Human umbilical vein endothelial cells (HUVECs) will be cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin in humidified 100% air at 37 °C with 5% CO_2. Confluent cell cultures of HUVECs will be incubated with ECM. To investigate the role Nedd4, we will knockdown and overexpress Nedd4 in these cells. siRNA will be used to knockdown the expression of Nedd4 using the Lipofectamine™ 2000 transfection reagent, following the manufacturer's instructions. The siRNA (sense, 5′ to 3′) sequence targeting respective human mRNA will be

Nedd4: 5′-UAGAGCCUGGCUGGGUUGUUU-3′

Over expression of Nedd4 will be obtained by transfection of pcDNA3.1- Nedd4 into the HUVECs. The cells will be transfected as discussed and then incubated with siRNA or pcDNA3.1- Nedd4 for 24 hours. Transfected HUVECs will be then stimulated with ox-LDL (100 µg/ml) (59) and palmitate (50 µg/ml) (62) for 24 h.

Lipid deposition:

To check the lipid deposition in HUVECs after Nedd4 inhibition and overexpression, the cells will be stained with 0.5% oil Red O solution for 1 h, and then differentiated in a 60% propylene glycol solution for 5 min to confirm the lipid deposition.

b. Quantitative Real-Time PCR analysis:

RT-PCR will be performed to determine the mRNA expression of Nedd4, IGPR-1, Caspase- 4/5 and GSDM-D, using gene specific primers. The primer sequences that will be used are as follows:

Nedd4: Forward 5′-TTGCACTTTGCAGCCAGAAG -3′,

Reverse 5′-CTGTCTGGGAGTCAGCTTCA -3′

IGPR-1: Forward 5′-GACCCGAATTCAGAAACAC -3′,

Reverse 5′-GCCCTTCTCAAAACGTA -3′

CASP4: Forward 5′-AAGAGAAGCAACGTATGGCAGGAC-3′,

Reverse 5′-GGACAAAGCTTGAGGGCATCTGTA-3′

CASP5: Forward 5-GGTGAAAAACATGGGGAACTC-3,

Reverse 5′-TGAAGAACAGAAAGCAATGAAGT-3′

GSDM-D: Forward 5′- GTGTGTCAACCTGTCTATCAAGG-3′

Reverse 5′-CATGGCATCGTAGAAGTGGAAG-3′

Briefly, total mRNA will be extracted using TRIzol reagent (Invitrogen, catalogue no. 15596-026) and then reverse transcribed into cDNA. PCR amplification will be

performed with SYBR Green PCR Master Mix. GAPDH will be used as an internal control. Data will be presented as fold increase of target genes normalised to the housekeeping gene, GAPDH (64).

c. Western Blotting analysis:

Total cell lysates will be prepared and micro-centrifuged. Proteins in the supernatant will be extracted, fractionated using 12% SDS-PAGE, and transferred onto PVDF membranes (Millipore). Membranes will be blotted with primary antibodies against NEDD4, IGPR-1 caspase-4/5 and GSDM-D at 4°C over-night followed by incubation with secondary antibodies for 1 h at room temperature. The chemiluminescence technique using LI-COR Odyssey Fc will then be used for identification of bands, and the band intensity of target proteins. GAPDH will be used as a loading control (65).

d. *In vitro* IGPR-1 ubiquitination assay:

In vitro IGPR-1 ubiquitination assays will be performed using ubiquitination kit following the manufacturer's instructions. Briefly, the assay will be performed in 50 µl reaction volume with the following components: 2 µg of recombinant biotinylated ubiquitin, 2.5 µM E2 (a mixture of E2 enzymes: UbcH1, UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8, UbcH10, UbcH13/ Mms2) or 100 ng of purified GST-IGPR-1 and 1 µg GST-NEDD4. Following the reaction, the samples will be analysed by western blot via Streptavidin-HRP (37).

7. Novelty of the study

Epidemiology depicts that cardiovascular diseases lead to increased mortality rates globally causing serious threat to human health, and treatment of these diseases have become serious concern. Cardiovascular diseases are mostly determined by various vascular diseases, such as atherosclerosis and stroke. From last decade, vast experimental data have demonstrated the role of pyroptosis in atherosclerosis where

highly pro-inflammatory necrotic type of cell death occurs in various cells such as endothelial cells, macrophages, smooth muscle cells etc. It has been observed that Caspase/inflammasome pathway contributes majorly to this pro- inflammatory necrotic type of cell death. Recently, it has been observed that Gasdermin-D leads to pore formation in membrane of the endothelial cells which plays an important in the pyroptosis of these cells causing release of proinflammatory cytokines during atherosclerosis. Pharmacological inhibition targeting various mechanistic pathways of pyroptosis, such as NLRP3 assembly, GSDMD cleavage and pro- inflammatory cytokine release have been proven to be novel potential targets for ameliorating atherosclerosis. Therefore, the aim of the study is to elucidate the role of Nedd4/Caspase-4/5-GSDM-D signalling pathway during pyroptosis in atherosclerosis. Therefore, our study may be exploited to develop a novel therapeutic approach for prevention of atherosclerosis in this direction.

8. Expected Outcome

The importance of pyroptosis in atherosclerosis has garnered significant attention. The canonical inflammasome pathway is mainly responsible for pyroptosis-induced inflammation within atherosclerotic plaques. Multiple inhibitors of pyroptosis pathway like NLRP3, caspase-1 and gasdermin-D in CVD have been shown to inhibit cardiac inflammation and cardiac function. However, the processes driving pyroptosis and its regulatory role in atherosclerosis is still unclear. Present study will explore how Nedd4 regulates the non- canonical pyroptotic caspases 4/5 and cell adhesion molecule IGPR1 during atherosclerosis in ECs. Initially the project will verify whether caspase4/5-inflammasome activation could be targeted to inhibit the release of the proinflammatory cytokines IL-ß and IL-18 via GSDM-D during pyroptosis which cause an inflammatory cascade in the development of atherosclerosis. Furthermore, the study will be extended

to explore the role of Nedd4 in regulating the non-canonical pyroptotic signalling pathway and IGPR1 in ECs during atherosclerosis. If the data obtained is encouraging, Nedd4/Caspase-4/5-GSDM-D Signalling Pathway can be targeted to prevent the generation of inflammatory cascade during atherosclerosis. Understanding this mechanism and its role could facilitate the discovery of anti-inflammatory drugs which together with hypolipidemic drugs like statins may be exploited as potential therapeutic strategy that may be beneficial for prevention of atherosclerosis.

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